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Microbial water quality: Voltammetric detection of coliforms based on riboflavin–ferrocyanide redox couples

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ABSTRACT

The ability to screen water for the presence of faecal contamination is a pressing need for rural communities dependent upon local purification systems. While there are a multitude of coliform detection assays based on the activity of β-galactosidase, this report details the adaptation of a voltammetric pH sensing strategy which could offer rapid analysis. The approach exploits the bacterial metabolism of lactose via pyruvate to lactate with the subsequent decrease in pH measured by examining the peak separation of a riboflavin (sensing) – ferrocyanide (reference) couple. Disposable carbon fibre electrodes were used as *in situ* sensors in *Escherichia coli* cultures (10^3–10^7 cfu/mL) with detection times of 4 h enabling confirmation of coliform activity. The bacterial compatibility of the riboflavin–ferrocyanide system in combination with the simplicity of the detection methodology, stand in marked contrast to many existing coliform assays and could open new avenues through which voltammetric pH sensing could be employed.

1. Introduction

Access to safe drinking water has been a long-standing concern for those in low/middle income countries (LMIC) and is presently a core priority in the Sustainable Development Goals (SDGs) ratified by the UN General Assembly in 2015 [1,2]. While 91% of the world population presently acquires drinking water from improved sources, it has been estimated that some 10% of the latter remain contaminated with faecal material (at least 100 coliform bacteria per 100 mL) [3]. The provision of field diagnostics that can speedily identify faecal contamination of water could dramatically reduce the impact of diarrhoeal diseases where surveillance data indicate that almost half a million deaths occur annually in infants under five years of age [4]. Indicator species such as coliforms and *E. coli* are typically used as initial biomarkers of water quality with plate counts, fermentation tubes and membrane filtration serving as the standard approaches in their enumeration [5–8]. Unfortunately, the time lag between sampling and analysis can extend to 72 h and is clearly impractical when considering the needs of rural communities dependent on local purification systems. The aim of the present communication has been to investigate the development of a disposable electrode system that could be readily accessible to those in LMICs and provide speedier confirmation of microbial water quality.

The detection rationale centres on the activity of the β-galactosidase enzyme inherent to coliforms and *E. coli* [5]. The enzyme is responsible for the conversion of lactose to galactose and glucose with the latter being further metabolised by the cellular machinery to pyruvate. Under anaerobic conditions, the pyruvate is further degraded to lactate [9] and it was envisaged that the consequent decrease in pH from such processes (Fig. 1A) could be exploited as both an indicator of coliform presence and their relative concentration. Isopropyl-D-1-thiogalactopyranoside (IPTG), a molecular mimic of allolactose, is introduced to trigger the expression of the lac operon resulting in transcription of the *lacZ* gene encoding β-D-galactosidase [10]. While IPTG is not metabolised, its effect is to increase the amount of enzyme and thus offers a means of improving the sensitivity of the assay by increasing the metabolism of lactose and the production of acids. The foundations of such an approach have long historical roots dating back to the “Wurtz” method (c.1893) where samples were plated onto a litmus lactose medium whereby growth of the bacteria would induce a change in local pH and therein a change in colour on the plate [5]. The aim here is to utilise a voltammetric method to determine changes in pH brought

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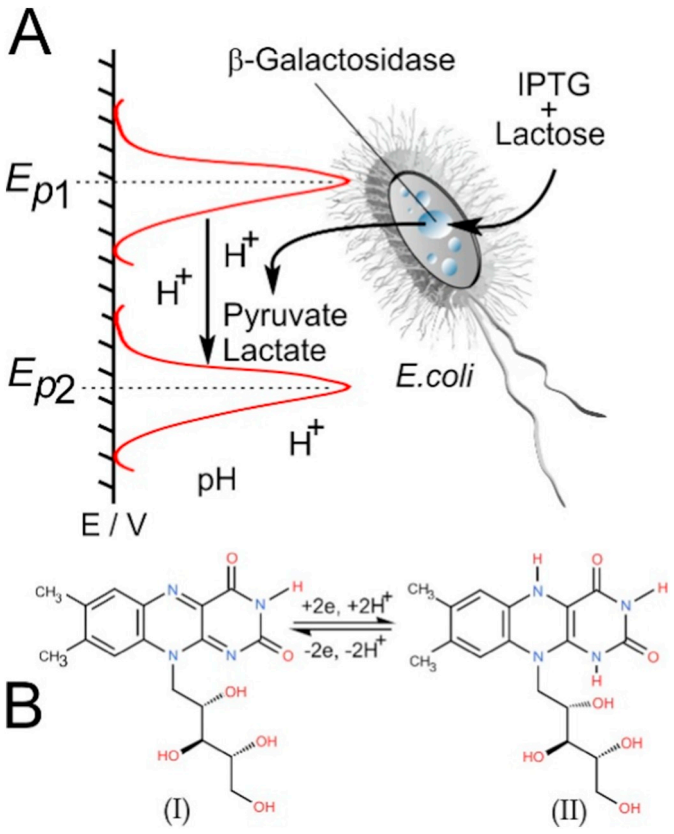


Fig. 1. A) Exploiting shifts in redox peak potentials (Ep_1 – Ep_2) arising from bacterial fermentation processes to detect the presence of coliforms. B) Riboflavin redox transitions.

about by bacterial metabolism, thereby avoiding the ambiguities of visual colour identification and speed identification.

Conventional pH electrodes are neither economically nor procedurally practical for implementation within LMIC contexts, and thus an alternative approach is required. There is a wealth of literature on the application of electroanalytical techniques to the detection of bacteria [11–14], but as yet few have examined the use of voltammetric pH measurement strategies. The latter tend to be based on the pH dependence of the peak process inherent to the oxidation/reduction of quinone type redox systems [15–19]. While the shift in peak position is used as an indirect marker of pH, none have yet been used for microbial water quality measurements. In the present instance, a riboflavin redox system is employed as the principal pH sensitive redox probe (Fig. 1B). In contrast to the quinone systems, it provides a low-cost biocompatible alternative that would incur no safety issues over handling or disposal – given its eventual application within a disposable sensor.

One issue that does arise, however, relates to the nature of the reference electrode. The latter is a critical component given that subtle changes in the peak position of the redox probe are used to determine pH (Fig. 1A, $Ep_1 \rightarrow Ep_2$). However, it could be envisaged that the ability of the bacteria to thrive, and hence actively metabolise lactose, would be compromised by the potential antimicrobial characteristics arising from the use of a conventional Ag|AgCl pseudo reference. The introduction of ferrocyanide, a pH-insensitive probe, was proposed as an internal reference system. Ferro/ferricyanide redox couples are widely used in respirometric assays where the microbial reduction of an electron acceptor ($Fe[CN]_6^{3-}$) can be related to the microbial metabolic activity and takes advantage of the low toxicity of the ferro/ferri couple [20]. As such, the latter should be capable of providing an unambiguous reference peak without unduly affecting the bacteria.

2. Experimental details

2.1. Materials and instrumentation

All chemicals were obtained from Sigma-Aldrich (Gillingham, UK), were the highest grade available and were used without further purification. Toray Carbon Fibre Paper (TGP-H-30) was purchased from E-TEK (USA) and used as received. Electrochemical analysis was carried out using an Anapot potentiostat (Zimmer & Peacock, Royston UK) with a standard three-electrode configuration with either a glassy carbon (3 mm diameter) or Toray carbon fibre mat (2 × 2 mm) as the working electrode. Platinum wire served as the counter electrode and a conventional silver/silver chloride (3 M KCl, BAS Technicol UK) half cell reference electrode unless otherwise specified. All measurements were conducted at 22 °C ± 2 °C. Carbon fibre electrodes were sealed within a polyester laminate as described previously [21] and pre-anodised in 0.1 M NaOH (+2 V, 5 min) [22].

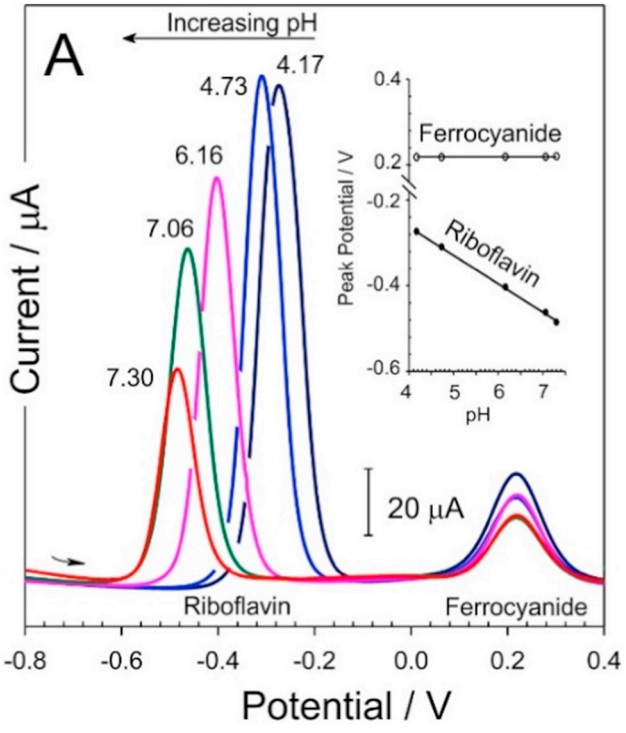
2.2. Bacterial culture

Escherichia coli (K12 strain) was grown from an existing stock (previously stored at −80 °C, 25% glycerol) by streaking onto a Luria Bertani (LB) agar plate containing tryptone (10 g/L), yeast extract (5 g/L) and NaCl (10 g/L) and agar (15 g/L). A single colony was transferred from the stock plate to a 50 mL falcon tube and incubated at 37 °C for 18 h at 150 rpm. Overnight cultures were sub-cultured to an OD_{600} of 0.4 before undergoing an 8 fold serial dilution. Lactose (provided at either 1% or 5% w/v), riboflavin (10 μM), ferrocyanide (500 μM), and IPTG (500 μM) were added to working stocks of *E. coli* (10^3, 10^5 and 10^7 cfu/mL) and incubated at 37 °C. Test samples (2 mL) were analysed using square wave voltammetry every hour over a 5 h period with a final measurement taken after 24 h.

3. Results and discussion

Square wave voltammograms detailing the response of the carbon fibre electrode towards riboflavin and ferrocyanide (vs. a Ag|AgCl reference) in buffers of varying pH are detailed in Fig. 2A. The peak position of the ferrocyanide, as expected, remains constant while the riboflavin moves to less negative potentials with decreasing pH. Similar observations were made by Hegarty and colleagues with a custom synthesised polyflavin system [23]. Ferrocene derivatives have been used in a similar vein to ferrocyanide as a pH insensitive reference [15, 17, 19], but the water solubility and low toxicity of the latter are key advantages here [20]. The change in the riboflavin oxidation potential is highlighted in the inset within Fig. 2A. Each point represents the average of 3 measurements and, as the error is less ± 0.7 mV, the error bars are indistinguishable from the marker. The electrode-redox probe response was found to exhibit near Nernstian behaviour (Ep_a/V = −0.067 pH − 0.041; R^2 = 0.999; N = 12) over pH 4 to pH 7. It should be noted that the riboflavin peak diminishes in magnitude beyond pH 7 and deviates from linearity above pH 8. While this can, at first, appear as a considerable limitation, it is necessary to note that *E. coli* thrives within a narrow pH range and, under the conditions of this assay, the pH transition was typically from pH 6.8 decreasing to pH 5 (after 24 h). As such, the linear range is commensurate with the present application. The pH response of the flavin was similar to those observed with quinone type species but it is notable, from the perspective of the intended application, that the measurements were conducted without degassing the solution. There is no discernible interference from oxygen despite the flavin redox peak residing in a cathodic region.

The response of the electrode in the presence of *E. coli* (10^7 cfu/mL) is considered in Fig. 2B. Square wave voltammograms were recorded at various time intervals over a 24 h period. In this case, the electrode configuration was modified to utilise carbon fibre as the combined



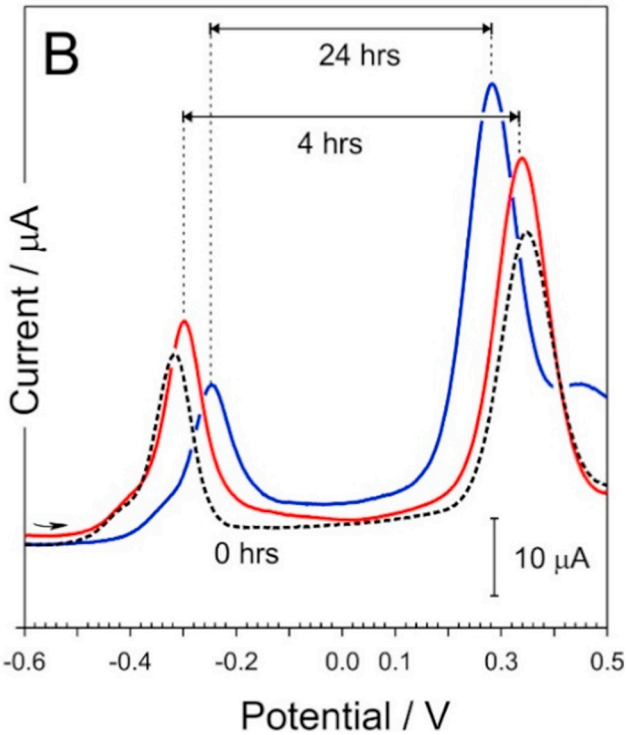


Fig. 2. Square wave voltammograms detailing the response of an anodised carbon fibre electrode towards riboflavin (10 µM) and ferrocyanide (500 µM) in (A) buffer solutions of varying pH and (B) an *E. coli* (10^7 cfu) suspension over a period of 24 h.

reference/counter electrode. In the absence of a defined redox potential at the reference electrode, the peak positions of the flavin and ferrocyanide can be seen to vary between scans. In this instance, the ferrocyanide peak position now becomes the internal reference from which the separation of the flavin peak position ($Ep_{\text{riboflavin}} - Ep_{\text{ferrocyanide}}$) is

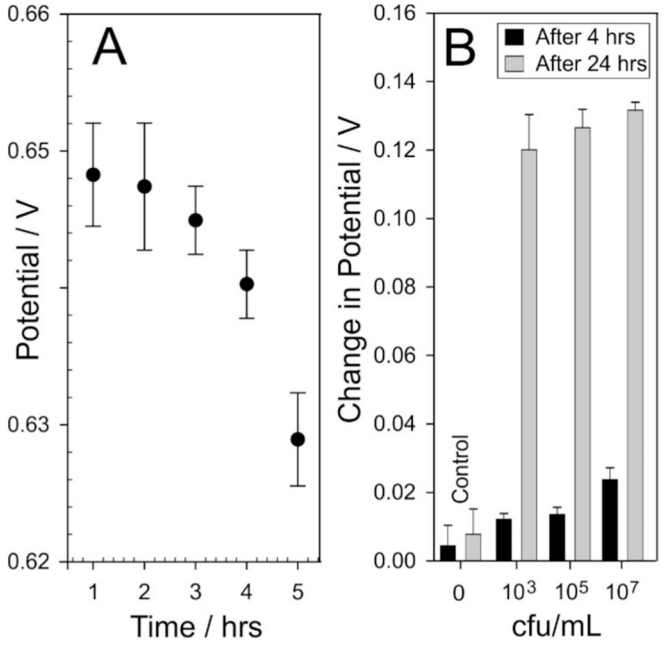


Fig. 3. A) Influence of increasing fermentation time on peak potential separation ($Ep_{\text{riboflavin}} - Ep_{\text{ferrocyanide}}$) recorded in *E. coli* (10^7 cfu/mL) samples. B) Overall change in potential ($\Delta E_t - \Delta E_{t0}$) for different *E.coli* samples after 4 h and 24 h.

measured. Inspection of the relative peak position of the two probes indicates a narrowing of the separation between the two with increasing incubation periods. This would be consistent with the pH of the medium decreasing (cf. Fig. 2A) as the bacteria progressively metabolise the lactose. It should be noted that the scan range is minimised in order to reduce the possibility of oxidising other electroactive components within the culture medium. In this case, the bacteria are cultured in LB broth which contains a tryptone digest containing tryptophan and tyrosine whose oxidation at the higher potentials (typically > +0.6 V) would otherwise foul the electrode. Neither the riboflavin nor the ferrocyanide was found to have any significant impact on the growth of the bacteria over the 24 h duration. It is also important to note that the peaks are unambiguous and devoid of any competing processes present in the culture media.

A more quantitative inspection is shown in Fig. 3A for the 10^7 cfu/mL culture where distinct changes in the peak separation, and hence medium pH, can be seen after the first few hours. The sensitivity of the approach was also assessed with *E. coli* at 10^3 cfu/mL and 10^5 cfu/mL and the change in potential from the initial electrode measurement after 4 h and 24 h are compared in Fig. 3B. Clearly, the longer the culture is incubated, the greater the change in the pH, the greater the shift in riboflavin peak potential and hence the greater the change in potential from the initial/baseline setting. As expected, there is a marked difference in the rate at which the pH changes across the different *E. coli* cell concentrations with the 10^7 cfu/mL samples effecting a much more pronounced/sustained change within a few hours. Inspection of Fig. 3B, however, reveals that after 24 h, the difference in potential observed for each culture was more or less similar. The change in potential (from initial to the final 24 h) typically represents a 120 mV difference which is equivalent to a fall of almost 2 pH units. This was corroborated through measuring the pH with a conventional pH probe where the initial and final pH values of the medium were found to be 6.8 and 5 respectively. The levelling of the pH at around pH 5 was attributed to the decrease in the prevailing pH of the medium becoming less favourable for the continued growth of the bacteria. Increasing the concentration of lactose (from 1% to 5% w/v) did not

yield any significant change in the final pH from that observed with the lower lactose concentration and suggested that nutrient supply was not a limiting factor.

There has been extensive interest in the development of coliform detection assays that exploit the activity of the β-galactosidase enzyme and these almost invariably rely on the enzymatic cleavage of modified substrates containing either a chromogen (i.e. chlorophenol red) [24] or an electroactive label (i.e. *p*-aminophenol) [25,26]. Electroanalytical systems have employed numerous material modifications to the electrode surface and associated protocols to enhance the detection of the released redox label and have included: carbon nanotubes [27], bacteriophages [25,26], tyrosinase [27,28] or complex redox cycling configurations [29]. The incubation period can vary from 4 h through to 10 h but, it should be noted that, the complexity of the sensor/assay design would be a major hurdle in the pursuit of a disposable and economically viable system suitable for implementation in a LMIC context [26–29]. The approach taken here, in its simplest form, offers a turnaround time of just 4 h which is competitive with the more complex systems employing labelled substrate β-galactosidase assays. Fast turnaround times are an imperative where reducing the analysis time can greatly reduce the likelihood of communities using unsafe water.

The summary data for the 10^3 cfu/mL samples highlight the fact that the 4 h analysis period is near the detection limit (based on 3:1 signal/control ratio), but it could be anticipated that further gains in performance could be achieved where preconcentration (through filtration or centrifugation) of the sample is employed prior to analysis. This could offer a route through which the gold standard of 1 cfu/mL detection limit could be obtained. The approach advocated here has a safeguard through exploiting the sustained change in pH as a detection signal. The latter can only arise as a consequence of the cumulative activity of the β-galactosidase enzyme and thus, rather than use a single/discrete measurement event, the *in situ* placement of the electrodes during incubation, as indicated in Fig. 3A, can enable the progress of the assay to be monitored. Moreover, the approach can also enable discrimination between viable and non-viable coliforms where the growth of the bacteria, in combination with the added IPTG, facilitate increased enzyme molecule numbers and hence increase the speed at which pH changes. While it could be envisaged that some treatment processes may be successful in killing the coliform species yet release active galactosidase, the low concentration of the latter (relative to an ever increasing pool encountered in a growing bacterial population) would yield minimal change in pH. Furthermore, the combination of the detection approach with sample filtration would effectively remove any residual, extracellular β-galactosidase.

4. Conclusions

Voltammetric approaches to the measurement of pH have received considerable attention in recent years, but have largely focused on the material modification of the electrodes and the subsequent elucidation of the underlying mechanism. The approach taken here highlights how the methodology could be applied within microbial water quality assessment. The latter has long been problematic from an electroanalytical perspective. While ever more complex electrode modifications have been proffered as potential solutions, it is possible that simplicity may be the more profitable route. This is especially pertinent in the screening of water quality where there is a need for low cost devices produced in large volume. Cost and affordability of the eventual device are highly pertinent factors in the present context where some 30% of the world's population is estimated to subsist on less than $2 per day. The carbon fibre system outlined here does not aim to highlight an end-product, but rather highlights a new direction in which advances in voltammetric pH sensing could be trained and enable further evolution towards an accessible device for LMIC use.

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